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pH dependent spectral properties and electronic structure of benzothiazol containing cyanine dyes

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The absorption and emission properties of some cyanine dyes over a range of 8 pH units are presented. Theoretical investigations of their electronic structure in the ground and excited states have been undertaken and the nature of the electronic transitions in the protonated and non-protonated forms is rationalised.

1. Introduction

The use of fluorescent labeling reagents and in particular the use of cyanine dyes for biological applications have expanded rapidly in recent years [1–3]. The excitation and emission spectra of these probes span the visible and NIR spectra from 450 nm, where autofluorescence from biomolecules is greatly reduced, up to 800 nm. These fluorescent probes have wide-ranging applications, including DNA sequencing, flow cytometry and high throughput screening [4]. Biochemical processes frequently involve protonation and deprotonation of biomolecules with concomitant changes in the pH of the medium. Consequently, there has been an increase in the use of pH-sensitive fluorescent probes for intracellular studies [5]. It is now of theoretical and practical interests to investigate the halochromism of cyanine dyes.

In this paper, we present the absorption and emission properties of some cyanine dyes over a range of 8 pH units. The interaction of protons with multiple sites of different characters on the chromophore results in changes of the absorption as well as the emission spectra.

To understand better changing spectral properties of studied cyanines under protonation, we have carried out theoretical investigations of their electronic structure in the ground and excited states and also determined the nature of electronic transitions protonized and non-protonized protolytic forms.

2. Results and discussion

2.1. Spectral properties of cyanines

Our studies were based on two cyanines: 3-(carboxyethyl)-2-[(1*E*,3*E*)-3-[3-(carboxyethyl)-2(3*H*)-benzothiazolylidene]-1-propenyl] benzothiazolium iodide (**1**) and 3-(carboxyethyl)-2-[(1*E*,3*E*,5*E*)-5-[3-(carboxyethyl)-2(3*H*)-benzothiazolylidene]-1,3-pentadienyl] benzothiazolium iodide (**2**) depicted on Scheme 1. The first of these cyanines was also presented by its ester form **1a**, where $R = CH_3$.

The absorption band maximum of the cyanine **1** (λ_{abs}) was detected at 558 nm. From Fig. 1, it is obvious that as the solution becomes more acidic after addition of HCl acid, we observe a small hypochromic effect. It is interesting to notice the systematic shoulder on the characteristic absorption band of our product. As given in the literature [6], we are allowed to attribute the absorption band at 558 nm to the non-associated form, and the systematic shoulder at 522 nm – to the aggregate form of the molecules. However, taking into account the high symmetry of absorption and fluorescence spectra, and the presence of long-wavelength shoulder on the emission band, it is also possible to assign the observed shoulders to vibronic component of the main bands [7,8].

The maximum of the fluorescence (λ_{fl}) excited at 530 nm was observed at 579 nm. The value of the Stokes shift $\Delta\nu_{St}$ was

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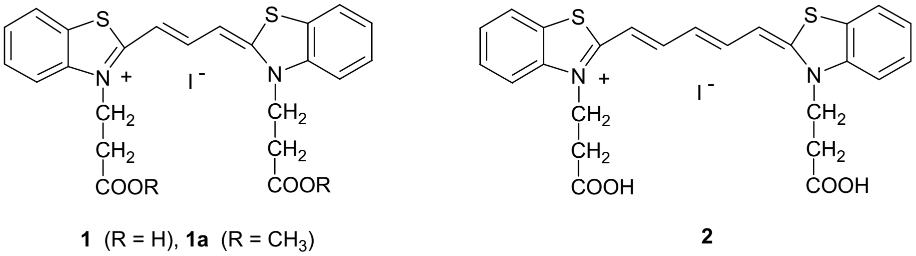


Scheme 1. Synthetised cyanines dyes.

1600 cm^{-1} that is typical for dyes having rigid molecular structure. Increase of the acidity results in the diminution of fluorescence intensity.

The same spectral behaviour was detected in the case of dimethyl ester **1a**. The changes in the absorption and fluorescence spectra are depicted in Fig. 2. It means that there is no significant influence of carboxyl fragment on cyanine chromophore.

Somewhat higher sensitivity of the fluorescence of **1** to pH changing in comparison with **1a** is probably due to some sterical effects produced by substituted carboxyl fragments under the structural relaxation upon the excitation.

The lengthening of the polymethine chain in the case of **2** leads to bathochromic shift of long-wavelength absorption band up to 665 nm. The difference in λ_{abs} of **1** and **2** is approximatively 2290 cm^{-1} (90 nm) that is typical when addition of a double bond in the chain of cyanine dyes [9,10].

The maximum of fluorescence of **2** has been detected at 672 nm, and the Stokes shift is very small – only 160 cm^{-1}. The difference between emission band maxima of **1** and **2** is 2390 cm^{-1}, i.e. it corresponds to the shift of absorption bands.

The increase of solution acidity results in a very important changes of spectral properties of **2**. Fig. 3 shows that the increase of the solution acidity leads to a drastic decrease of absorption and to total disappearance of fluorescence. It is obvious that these spectral effects are due to protonation of nitrogen atom and to formation of di-cation. However, it is remarkable that the important spectral effects have been obtained only for **2** with longer polymethine chain.

These experiments show that the equilibrium described in Scheme 2 is efficient towards absorption and fluorescence only with an electronic delocalization including nine atoms. When the two nitrogen atoms are supporting a positive charge (no more possibility of delocalization of the electronic pair), the intensity is decreasing. It is also noteworthy that the carboxylic moiety has apparently no influence on this phenomenon.

To study these experimental facts, calculations concerning electronic structure of these compounds were carried out.

2.2. The electronic structure and the nature of electronic transitions of 1 and 2

The analysis of the data listed in Table 1 demonstrates that **1**, **1a** and **2** have symmetrical re-distribution of electronic density in the ground state. The positive charge of the cations is localized on heterocyclic fragments (fragments B and C on Scheme 3), whereas the polymethine chain (fragment A) is even slightly negatively charged. The numbers σ, which reflect the differences between orders of bonds, are low, that indicate an important delocalization of π-charge in the polymethine chain.

The lengthening of the chain results in greater delocalization of the positive charge in the molecule **2** (decreasing positive charge on benzothiazole fragments and decreasing negative charge on the chain). The σ value is some higher, that indicates a greater alternation of bond orders in the chain.

The excitation of **1** and **2** leads to following delocalization of the positive charge. The values of a charge transfer (Δq) from the heterocyclic fragments to the chain for **1** and **2** are 4–5% and 5–10%, correspondingly. The charge transfer upon excitation seems to be slightly asymmetric. This may be explained as by splitting of two degenerated excited states (S_1 and S_2), as by calculation error due to insufficient size of configuration interaction matrix.

The numbers of charge transfer $l_{B\rightarrow A}$ and $l_{C\rightarrow A}$ (Table 2) obtained by CNDO/S method demonstrate the same transfer of π-charge in the molecules **1** and **2** upon excitation. For $S_0 \rightarrow S_1$ transitions, these values are ~24%.

As it follows from values δ in Table 1, the excitation does not lead to significant alternation nor leveling of bond orders in the polymethine chain.

In Table 2, we can see results of calculations of absorption spectra by CNDO/S method. The obtained theoretical spectra are

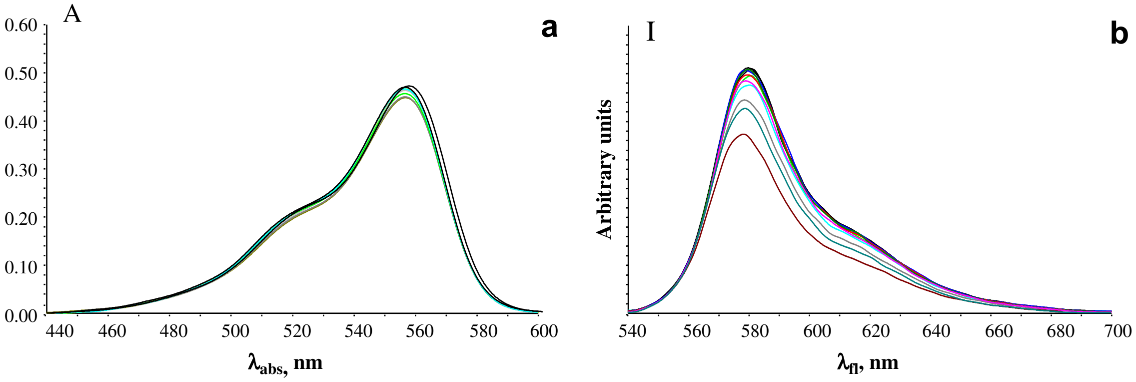


Fig. 1. Absorption (a) and fluorescence (b) spectra of the cyanine **1** at pH between 1 (lowest curve) and 10 (highest curve) in methanol–water mixture (1:9 v/v); dye concentration is 4.30 10^{-6} M.

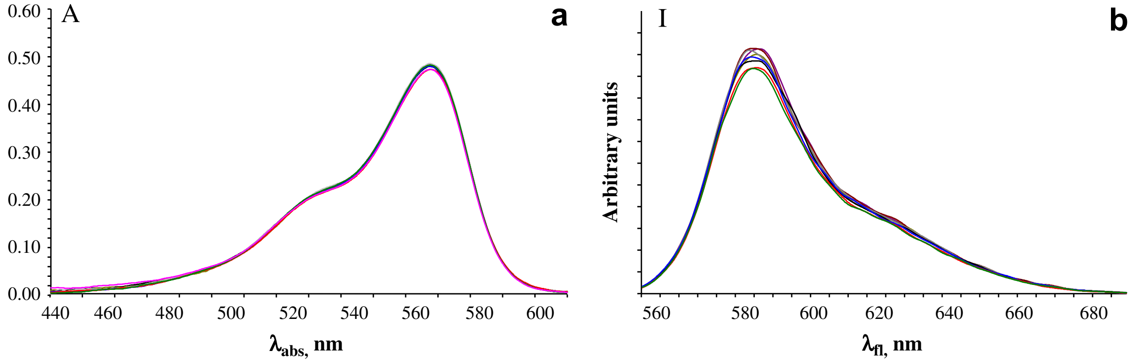


Fig. 2. Absorption (a) and fluorescence (b) spectra of the cyanine **1a** at pH between 1 (lowest curve) and 10 (highest curve) in methanol–water mixture (1:9 v/v); dye concentration is 1.14 10^{-6} M.

close to the experimental ones. According to calculations, there are three electronic transitions in the visible wavelength range. The transition $S_0 \rightarrow S_1$ of very high intensity corresponds to long-wavelength band, and then there are two forbidden transitions, invisible in experimental spectra. The next intense transition $S_0 \rightarrow S_4$ is at 314 nm (**1**) or 335 nm (**2**), i.e. outside of wavelength range under investigation.

All the electronic transitions are due to a chromophore localized on whole cyanine molecule. This conclusion may be done based on the values of excitation localization numbers, which are high as for heterocyclic fragments, as for polymethine chain (Table 2).

Theoretical positions of emission bands, obtained by CNDO/S calculations of molecules **1** and **2** with optimized S_1 state geometry, have worse correlation with experimental values. However, our calculations predict very low values of Stokes shifts of fluorescence corresponding to the ones observed earlier in experiment.

Protonation of nitrogen atom in the fragment C of the studied cyanine dyes leads to formation di-cations having another electronic density re-distribution and spectral properties. According to the data in Table 1, we can establish an asymmetric delocalization of the positive charge, and, consequently, nonequivalence of benzothiazole fragments in protonized cyanines. Moreover, protonized dyes have a very high alternation of bond orders in the chain (σ is 74.2×10^{-2} and 90.1×10^{-2}, correspondingly), close to maximal values (1.0×10^{-1} for **1** and 1.2×10^{-1} for **2**), when there is a simple alternation of single and double bonds.

The excitation of protonized **1** and **2** is accompanied by nonequivalent interfragmental charge transfer, which, however, results in sufficient regrouping (δ are 72.0×10^{-2} (**1**) and 68.2×10^{-2} (**2**)) and leveling (δ are 6.7×10^{-2} (**1**) and 14.6×10^{-2} (**2**)) of bond orders in the chain.

The nonequivalence of the benzothiazole fragments in the protonized dyes influences on the nature of electronic transitions in absorption spectra. According to data listed in Table 2, the long-wavelength $S_0 \rightarrow S_1$ transition is localized on the non-protonized benzothiazole fragment and on the chain ($L_A + L_B$ is equal to 93–95%) and is accompanied by π-charge transfer between A and B ($l_{B\rightarrow A}$ is 46% for **1** and 30% for **2**). The second forbidden transition $S_0 \rightarrow S_2$ is localized on the fragments A and C and is accompanied by charge transfer between them ($L_A + L_C$ is 85% and $l_{C\rightarrow A}$ is 44% for **1**, and $L_A + L_C = 92\%$ and $l_{C\rightarrow A} = 52\%$ for **2**). The next transition, which is outside of region 400–600 nm, is localized on the whole molecule of the dyes; thus, its nature is similar to one of the non-protonized forms.

The difference between energies of $S_0 \rightarrow S_1$ transitions of protonized **1** and **2** is lower than in the case of non-protonized forms. It seems to us that this effect could be explained by decreasing the size of corresponding chromophore upon protonation.

As it was mentioned above, the dyes **1** and **1a** do not sufficiently change their absorption in interval pH: 1–8. According the theoretical spectra, the long-wavelength band of protonized form of compound **2** is expected at 463 nm, and corresponding band in the experimental absorption spectra was detected at 470 nm (i.e. outside the wavelength range depicted in Fig. 3A).

According to our calculations, the theoretical emission bands of protonized **1** and **2** are at 620–640 nm and have very low oscillator strength, which could indicate the absence of the fluorescence. This

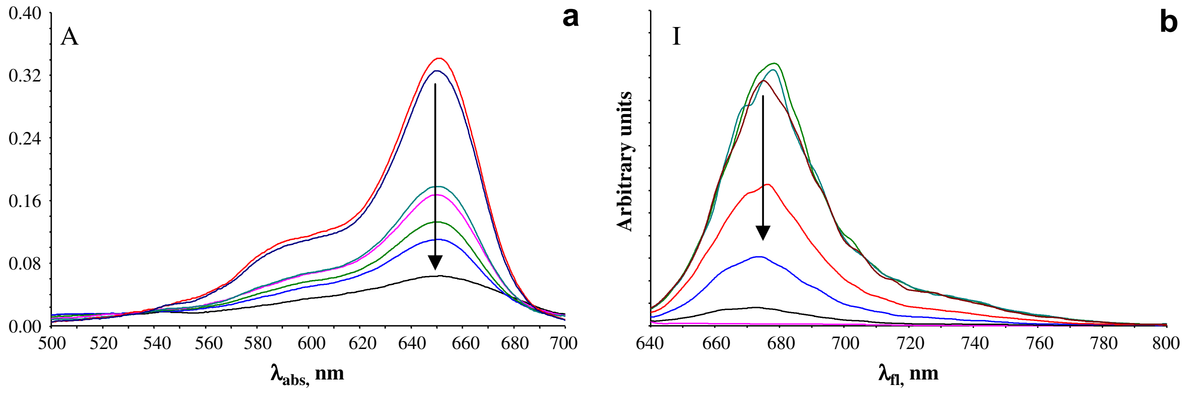
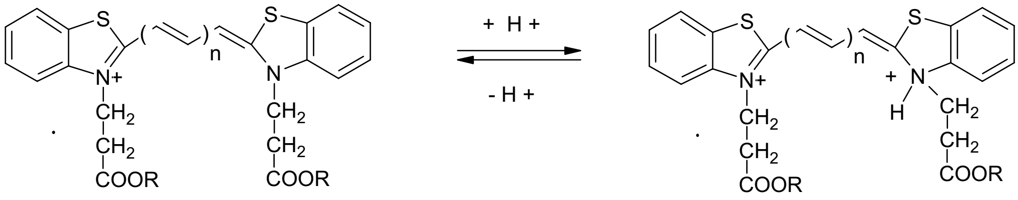


Fig. 3. Absorption (a) and fluorescence (b) spectra of the cyanine **2** at pH between 1 (lowest curve) and 10 (highest curve) in methanol–water mixture (1:9 v/v); dye concentration is 4.11 10^{-6} M.



Scheme 2. Acido-basic equilibrium of cyanine dye protolytic forms.

Table 1
Mulliken charges and bond order leveling in the ground and excited states obtained by AM1 and AM1/CI methods[a]

Compound	State	Charge localization					Charge transfer upon excitation[c] (Δq)		Bond order leveling	Changing of bond order leveling upon excitation
		Fragment B	Nitrogen atom in fragment B	Fragment C[b]	Nitrogen atom in fragment C [b]	Chain (fragment A)	B → A	C → A	$\sigma \times 10^2$	$\delta \times 10^2$
1	S_0	+0.551	−0.142	+0.551	−0.142	−0.101	+0.040	+0.053	7.5	5.5
	S_1	+0.471	−0.101	+0.458	−0.117	+0.071			7.1	
1 protonized	S_0	+0.877	−0.043	+0.920	+0.093	+0.204	+0.175	+0.017	74.2	72.0
	S_1	+0.702	−0.066	+0.903	+0.127	+0.395			6.7	
2	S_0	+0.524	−0.148	+0.524	−0.148	−0.048	+0.049	+0.103	11.5	4.7
	S_1	+0.474	−0.089	+0.419	−0.153	+0.104			8.9	
2 protonized	S_0	+0.844	−0.054	+0.849	+0.098	+0.307	+0.175	−0.038	90.1	68.2
	S_1	+0.669	−0.093	+0.877	+0.124	+0.454			14.6	

[a] The localization and labeling of molecule fragments are indicated in Scheme 2.
[b] Fragment/atom joining proton upon protonation process.
[c] Transfer of the positive charge is indicated.

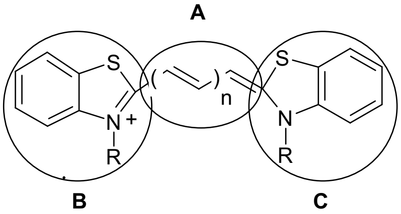
prediction can explain the decrease of emission intensity of **1** and **1a** and disappearance of fluorescence of **2** under protonation.

2.3. Acid–base properties of **1** and **2**

It is evident that drastic differences in spectral behaviour of **1** and **2** in acidic media are due to basicity of the dyes, which depends on the length of the polymethine chain. The lengthening of the chain leads to delocalization of the positive charge on the molecule, which results in the increase of negative charge on a basic centre (nitrogen atom in benzothiazole fragment C) and in the stabilization of protonized forms of the cyanine dyes.

It is known that even the small growth of negative charge on the basic atoms leads to the sufficient increase of basicity of compounds and, on the contrary, the small decrease of negative charge results in the great increase of acidity. Thus, in the case of oxygen containing compounds, it was demonstrated [11] that changing the charge $\Delta q \sim 0.07e^-$ results in changing equilibrium constants for 10.3 orders of magnitude. According to Table 1, the value of negative charge on the basic nitrogen atom on going from **1** to **2** increases in 0.006 that could correspond to ΔpK_b close to 1.

To estimate the influence of the polymethine chain length on the basicity of **1** and **2**, we calculated approximate values of pK_b using ACD Labs 4.56 Program Package [12]. This program permits to calculate theoretical pK_b close to experimental values as for thiazole (experimental – 2.53 [13] and calculated – 2.44 ± 0.5) and 2-aminothiazole (experimental – 5.39 [13] and calculated – 5.42 ± 0.5), as for complex derivatives of thiazole – 3-thiazolylchromones [14].



Scheme 3. The fragments selected for the calculation of the repartition of the electronic density.

Using ACD Labs, we calculated pK_b values not only for **1** and **2**, but also for their homologues having smaller and greater lengths of polymethine chain. The obtained results are listed in Table 3.

We can see that the calculated pK_b values change sufficiently for $n = 0$–2, and the following lengthening of the chain does not lead to additional delocalization of the positive charge, and thus, it does not influence on the basicity of the dyes.

The data in Table 3 demonstrate a linear dependence between pK_b values and charges on nitrogen atom in benzothiazole fragment C. The corresponding plot is depicted in Fig. 4. Using dependence between pK_b and q_N we can conclude that the increase q_N for $0.01e^-$ results to growth K_b of cyanine dye for 1.1 order of magnitude.

The obtained data permit to predict the possibility of using studied cyanine dyes as acid–base indicators. As it follows from Table 3, the best indicators for diapason pH 1–8 are derivatives of compound **2** and of homologous dyes with greater length of polymethine chain. The proposed indicators could be used not only for visual and photometric detection of solution acidity, but also for fluorimetric detection: changing pH value may be observed by fluorescence intensity.

3. Conclusion

The experimental absorption and fluorescence studies of cyanines **1** and **2** and of the methyl ester derivate (**1a**) in different pH media and calculatory studies of the electronic densities permit to conclude that application of even *N*-substituted benzothiazol cyanines can be used as acid–base indicators. Nethertheless, this application is only possible for a polymethine chain containing nine atoms. First investigations concerning reversibility of this phenomenon have been performed with cyanine **2**. The colour of

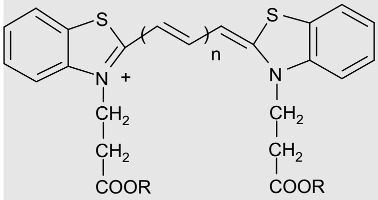
Table 2
Theoretical parameters of electronic transitions of **1**, **2** and their protonized forms obtained by CNDO/S method

Compound	Absorption spectra									Fluorescence spectra	
	Transition number	λ_{abs} (f), nm	C.I.[a], $d_{ij}^2\ \chi^2{}_{i\to j'}$	L_A, %	L_B, %	L_C, %	$l_{B\to A}$, %	$l_{C\to A}$, %	$l_{B\to C}$, %	λ_{fl} (f), nm	$\Delta\nu_{St}$, cm^{-1}
1	I	568 ($\sim$1.0)	$0.96\chi^2{}_{1\to 1'}$	35	30	35	24	24	8	590	690
	II	423 (0.000)	$0.96\chi^2{}_{2\to 1'}$	37	26	37	26	26	9		
	III	422 (0.000)	$0.94\chi^2{}_{3\to 1'}$	35	29	35	24	24	8		
	IV	314 (0.207)	$0.94\chi^2{}_{4\to 1'}$	33	34	33	20	20	9		
1 protonized	I	467 (0.778)	$0.85\chi^2{}_{1\to 1'}$	24	71	5	46	–	10	640	5740
	II	405 (0.003)	$0.94\chi^2{}_{2\to 1'}$	22	14	63	–	44	–		
	III	353 (0.273)	$0.83\chi^2{}_{3\to 1'}$	32	41	27	20	15	6		
2	I	627 ($\sim$1.0)	$0.92\chi^2{}_{1\to 1'}$	28	44	28	24	24	–	640	280
	II	427 (0.000)	$0.94\chi^2{}_{2\to 1'}$	32	35	33	32	31	7		
	III	427 (0.000)	$0.92\chi^2{}_{3\to 1'}$	33	34	33	30	31	11		
	IV	335 (0.349)	$0.94\chi^2{}_{4\to 1'}$	30	40	30	25	25	–		
2 protonized	I	463 ($\sim$1.0)	$0.62\chi^2{}_{1\to 1'} + 0.32\ \chi^2{}_{2\to 1'}$	63	30	7	30	10	9	620	5420
	II	400 (0.003)	$0.96\chi^2{}_{3\to 1'}$	26	8	66	–	52	–		
	III	351 (0.193)	$0.32\chi^2{}_{1\to 1'} + 0.61\ \chi^2{}_{2\to 1'}$	40	40	20	57	–	14		

λ_{abs}, λ_{fl} – wavelength of absorption (abs) and fluorescence (fl) maxima; f – oscillator strength; $\Delta\nu_{St}$ – Stokes shift of fluorescence; L_A, L_B, L_C – numbers of excitation localization on fragments A–C (see Scheme 2); lA → B and other similar labels are numbers of charge transfer between correspondent molecular fragments (only $l > 5\%$ are listed).

[a] Predominant configurations in C.I. matrix. An electronic transition is described through electronic configurations as $\Psi_k = \sum d_{ij}\chi_{i\to j'}$ (where $\chi_{i\to j'}$ is configuration describing a one-electron transition, and d_{ij} is power coefficient). d_{ij}^2 is probability of participation of a configuration in formation of given transition Ψ_k Only configurations with $d^2 > 0.2$ are listed.

Table 3
Theoretical pK_b values and Mulliken charges on basic nitrogen atom (q_N) for **1**, **1a**, **2** and model compounds

	n	Compound	pK_b (N) [±0.20]	pK_b (COOH) [±0.20]	q_N
	0		−5.31	3.61, 4.41	−0.146
	1	**1**	−4.23	3.61, 4.27	−0.154
	1	**1a**	−4.23	–	−0.153
	2	**2**	−2.42	3.61, 4.41	−0.167
	3		−2.78	3.61, 4.34	−0.171
	4		−2.29	3.61, 4.39	−0.172

the solution depends on the pH; the protonation of the nitrogen atom (acidic medium) induces the complete disappearance of the charge transfer responsible for the absorption band at 665 nm; if base is added to this solution, the solution regains its original pale blue colour. These first experiments need to be performed to confirm the potentiality of this cyanine as a good candidate to control pH media but also to follow evolution of pH in complex media, such as biological ones.

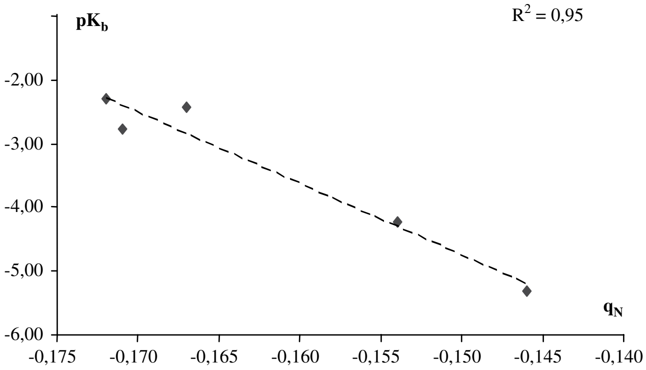


Fig. 4. The plot pK_b of benzothiazole containing cyanine dyes against the charge value on basic centre – nitrogen atom in benzothiazole fragment C.

4. Experimental

4.1. Apparatus

The UV spectra were measured on the *Perkin Elmer Lambda 9 UV/Vis/NIR spectrophotometer* at a temperature of 25 °C in the quartz cuvettes with a length of 1 cm.

The fluorescence spectra were measured on the *Perkin Elmer Luminescence spectrometer LS 50* at room temperature with the slits of excitation and of emission of 5 nm, in the quartz cuvettes with a length of 1 cm. The emission spectra were obtained, following a UV excitation corresponding to the maximum absorption of each product studied.

4.2. Preparation of buffer solutions

Stock pH solutions were prepared from the main buffer solution A, which is composed of H_3PO_4 (3.92 g), $B(OH)_3$ (2.47 g) and CH_3COOH (2.29 mL) made up to the 1 L mark in the volumetric flask with water. The pH of this solution was 1.81, and each buffer pH was made from this solution using a pH-metre and adding a different amount of NaOH (1 M) to the solution until the acquired pH was reached. Ten buffers with $1 < pH < 10$, which have been prepared, were then used to obtain solutions of cyanines **1**, **1a** and **2**.

4.3. Preparation of each pH cyanine solution

The protocol for the preparation of each different pH solution for each cyanine was the same using the different pH required each time. For example, for the preparation of cyanine **1** at $\mathrm{pH} = 2$ the procedure was as follows.

In a 50 mL volumetric flask, 25 mL of the buffer solution with $\mathrm{pH} = 2$ was pipetted into the flask, then 5 mL of NaCl (0.5 M) was pipetted into the flask and finally, 5 mL of the cyanine stock solution (4.3×10^{-5} M) was added and this solution was made up to the mark with water and inverted about 10 times to ensure a homogenous solution.

4.4. Quantum chemical calculations

Unconstrained geometry optimizations of isolated molecules in the ground (S_0) and excited singlet (S_1) electronic states, as well as calculation of Mulliken atomic charges, were carried out at the semiempirical AM1 (S_0 state) and AM1/CI (S_1 state) levels of theory [15] with standard procedures being employed together with the BFGS method [16–19] implemented in the MOPAC 2002 program package [20]. The final values of the energy gradient were always lower than 0.1 kcal/mol, and the eigenvalues of the Hessian matrix were all positive.

The wavelengths and oscillator strengths of electronic transitions, as well as numbers of excitation localization (L) and numbers of charge transfer (l), were calculated by CNDO/S method [21] using ground state (for $S_0 \rightarrow S_1$ transition) or excited state (for $S_1 \rightarrow S_0$ transition) geometries. Generally, 201 ground and single excited-state configurations due to excitation within the HOMO-10 and LUMO+10 molecular orbitals were used [22].

The estimation of $\mathrm{p}K_\mathrm{b}$ values was made using ACD Labs 4.56 Program Package [12].

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